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Review

# The zinc homeostasis network of land plants ☆

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ABSTRACT

The use of the essential element zinc (Zn) in the biochemistry of land plants is widespread, and thus comparable to that in other eukaryotes. Plants have evolved the ability to adjust to vast fluctuations in external Zn supply, and they can store considerable amounts of Zn inside cell vacuoles. Moreover, among plants there is overwhelming, but yet little explored, natural genetic diversity that phenotypically affects Zn homeostasis. This results in the ability of specific races or species to thrive in different soils ranging from extremely Zn-deficient to highly Zn-polluted. Zn homeostasis is maintained by a tightly regulated network of low-molecular-weight ligands, membrane transport and Zn-binding proteins, as well as regulators. Here we review Zn homeostasis of land plants largely based on the model plant *Arabidopsis thaliana*, for which our molecular understanding is most developed at present. There is some evidence for substantial conservation of Zn homeostasis networks among land pants, and this review can serve as a reference for future comparisons. Major progress has recently been made in our understanding of the regulation of transcriptional Zn deficiency responses and the role of the low-molecular-weight chelator nicotianamine in plant Zn homeostasis. Moreover, we have begun to understand how iron (Fe) and Zn homeostasis interact as a consequence of the chemical similarity between their divalent cations and the lack of specificity of the major root iron uptake transporter IRT1. The molecular analysis of Zn-hyperaccumulating plants reveals how metal homeostasis networks can be effectively modified. These insights are important for sustainable bio-fortification approaches. This article is part of a Special Issue entitled: Cell Biology of Metals.



## 1. Introduction

Zinc (Zn) is an essential element in all organisms. In its oxidized Zn(II)[1] form, which is the form of Zn found throughout biology, it acts as a catalytic or structural co-factor in a large number of enzymes and regulatory proteins [1]. Well-known examples in plants include the enzymes carbonic anhydrase and alcohol dehydrogenase, and the structural Zn-finger domains mediating DNA-binding of transcription factors and protein–protein interactions [2]. The use of a specific element in biological chemistry is a result of a complex interplay between various factors during evolution and under present-day environmental conditions. Among these, important factors are the bio-availability of the element for an organism, the efficacy and specificity of the element in fulfilling its various biochemical functions in comparison to elements that could be utilized alternatively, and constraints posed by the protein complement of the organism. Over the course of evolution, life has increasingly made use of Zn, with the Zn metalloproteome predicted to comprise around 5 to 6% of prokaryotic proteome and about 9% of the eukaryotic proteome [3].

Distinctive chemical properties make Zn a highly effective cofactor [4]. Among metals, Zn(II) is a strong and efficient Lewis acid catalyst and has very high binding affinity to a variety of ligands. Zn(II) is exceptionally flexible in the coordination geometries that it can adopt. Moreover, under biological conditions, Zn never undergoes changes in redox state so that it cannot directly participate in electron transfer reactions, for example in electron transport chains. When compared to transition metals that are able to accept or donate electrons themselves, such as Fe and Cu, the use of Zn is comparably safe in the proximity of sensitive macromolecules, in particular DNA, in the nucleus. The availability of Zn, which forms insoluble sulphides, has increased since the oxygenation of the Earth's atmosphere, and Zn concentrations are lower in aqueous environments than on land. This is probably the reason why the diatom, *Thalassiosira weissflogii* has evolved the ability to functionally replace Zn in carbonic anhydrase by Cadmium (Cd) when Zn is scarce, manifesting a Cd requirement that is unique in biology based on our present state of knowledge [5]. There is a pressing need for conclusive experimental approaches to determine the Zn metalloproteome in a number of organisms in order to experimentally validate *in silico* predictions based on the presence of amino acid sequence motifs.

☆ This article is part of a Special Issue entitled: Cell Biology of Metals.

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[1] $Zn^{2+}$ refers either to the "free" hydrated cation in solution or the cation alone. Elsewhere, Zn or Zn(II) are used synonymously, taking into account that the predominant proportion of Zn(II) is present either in complexed form bound to inorganic or organic ligands (e.g., inside cells) or in the form of insouble salts (e.g., in soil).

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Not surprisingly, as a consequence of the relatively abundant use of Zn in biological chemistry, Zn deficiency is a widespread condition. The WHO estimates that 31% of the world's population is at risk of Zn deficiency [6]. As there are no reliable biomarkers for Zn deficiency, its prevalence has long been underrated – different from Fe deficiency. Zn deficiency has a severe impact on public health and infant mortality, causing growth, cognitive and immune impairment, including enhanced susceptibility to diarrhea. In general, Zn deficiency is particularly widespread among the elderly, and it affects children most severely in regions of the world where the population relies primarily on cereal diets [7], which are poor in bioavailable Zn. Moreover, grain Zn concentrations have received little attention in breeding programs to date and have steadily decreased in cultivated wheat varieties since the green revolution [8]. In recent years, pronounced efforts have been made to increase Zn content and availability in staple crops (e.g., HarvestPlus, http://www.harvestplus.org/). These efforts are still at an early stage because the physiological and developmental processes controlling Zn accumulation in cereal grains, as well as the genes governing them, remain poorly understood [9]. Nevertheless, systematic efforts to screen natural genetic diversity for the purpose of introgressing identified target alleles are underway, and some pilot studies involving transgenic plants have yielded promising results [10,11] (see below).

A significant proportion of the Earth's arable land is considered Zn-deficient [12,13]. It is of paramount importance to take into account that Zn deficiency does not merely reduce crop quality and nutritive value. Micronutrient acquisition and micronutrient use efficiency are traits that critically affect crop yield [14,15] (see Zn deficiency symptoms below). This important fact has not received sufficient attention to date, given that there is a need to substantially increase crop yields in order to accommodate foreseeable population growth, increasing need for renewable non-food resources and the accelerating decrease in the area of arable land.

Although total Zn concentrations in eukaryotic cells are of the order of 100 μM, the internal concentration of free Zn is generally below the nanomolar range and, in *Escherichia coli*, below the femtomolar range [16]. Tightly controlling the concentrations and chemical speciation of intracellular Zn is a necessity for all organisms, because the binding of $Zn^{2+}$ to non-target sites would inevitably render these biologically non-functional. This has led to the evolution of a complex homeostatic network of Zn transporters, low-molecular-weight ligands and, although yet unknown in land plants, potentially also metallochaperone proteins that ensure the targeted delivery of the correct amounts of Zn to each apo-Zn protein, cellular compartment, cell, tissue and organ [17]. The concerted regulation of Zn homeostasis processes allows enhanced acquisition and redistribution of Zn, or storage and sequestration, in response to fluctuating environmental conditions and locally varying internal demands throughout the life cycle. How exactly this is accomplished remains poorly understood.

Various approaches have been developed in recent years with the aim of measuring cellular Zn concentrations *in vivo*. The Zn-fluorophore, Zinpyr-1, was used to image Zn within roots of *Arabidopsis thaliana*. As Zn-Zinpyr-1-dependent fluorescence is contingent upon the amount of Zn available for interaction with the fluorophore, comparisons of relative Zn levels between conditions and genotypes are possible [18]. However, the distribution of the fluorophore between and within cells cannot be fully controlled. The recent development of genetically encoded fluorescence resonance energy transfer-based ratiometric Zn sensors is very promising [19,20]. Using these sensors, dynamics of cytoplasmically available Zn concentrations were followed in mammalian cells. Estimates of resting cytoplasmic concentrations of 'free' Zn in mammalian cells between 5 pM and 1 nM were reported, with about 100-fold lower concentrations in ER and Golgi [19]. The targeting of sensors to specific intracellular compartments will help to experimentally address Zn concentrations at sub-cellular resolution. Cells control internal levels of Zn primarily by regulating transport processes that move Zn across membranes [21], with considerably less experimental evidence to date for the contribution of Zn binding. In most organisms, Zn is acquired either from the environment or from the diet by specific membrane transport proteins, sometimes operating in conjunction with chelators, which increase in abundance in response to Zn deficiency [22]. Work in yeast suggested that external Zn can change very rapidly, requiring the presence of proteins acting in Zn detoxification even in Zn-deficient cells [23]. In addition, transporters of divalent metal cations often exhibit broad substrate specificity, so that a deficiency in Cu, Fe or Mg may result in enhanced uptake and accumulation of toxic amounts of Zn. In these situations, Zn-specific transporters are needed in order to export excess Zn from the cytoplasm [24]. This has also been observed in plants [25]. In this review, we focus on the recent discoveries concerning Zn homeostasis in land plants, with a focus primarily on the model plant *A. thaliana* and closely related species.

## 2. Exceptionally large natural diversity in Zn homeostasis of land plants

Generally, plants exhibit Zn deficiency symptoms at shoot concentrations below a minimum of 15 to 20 mg Zn $kg^{-1}$ dry biomass. These symptoms include reduced biomass production, poor floral fertility, leaf chlorosis, increased shoot branching and early senescence of older leaves [15,26] (Talke & Krämer, unpublished observations). Zn deficiency leads to increased production of reactive oxygen species resulting from lowered Cu/Zn superoxide dismutase (CuZn-SOD) activity, the inhibition of protein synthesis and increased Fe accumulation. This causes damage to membranes, membrane proteins, chlorophyll and enzymes, resulting in leaf chlorosis and the inhibition of photosynthesis and growth [27]. Some plants are able to grow on highly Zn-deficient soils, but little is known about the genetic basis of this ability. Zn micronutrient use efficiency in wheat cultivars is not correlated with elevated rates of either root Zn uptake or translocation to the shoot, but instead with the ability to maintain activities of the Zn-dependent enzymes Cu/Zn-SOD and carbonic anhydrase even upon cultivation under Zn-deficient conditions [14].

Human activities such as mining, industrial contamination, sewage sludge amendments and agriculture have contributed to the pollution of large areas of agricultural soils with Zn. In addition, rarely occurring so-called calamine soils are naturally rich in Zn. While Zn contamination and the resulting bio-accumulation hardly ever reach levels that are toxic to mammals, Zn toxicity is a major environmental concern in other organisms. Importantly, the occurrence of Zn in minerals is usually linked with the occurrence of the chemically similar, highly toxic carcinogen cadmium (Cd), and often also of lead (Pb). The predominant threat for human health is Cd contamination and, in particular, its progressive accumulation along the food chain. Decontamination of Cd-polluted sites currently involves the expensive excavation of contaminated soil and disposal. One potential solution to this problem is phytoremediation, which aims to exploit the trait of hyperaccumulation of Zn and other metals found in a small number of plants. Zn hyperaccumulators, of which fourteen taxa are presently known, grow naturally on metalliferous soils and accumulate >1% (w/w) Zn in dry leaf tissues upon growth at their natural site [28,29]. The best studied Zn hyperaccumulators, *Arabidopsis halleri* and *Noccaea* (formerly *Thlaspi*) *caerulescens*, are also hyperaccumulators of Cd, i.e., capable of leaf accumulation of >0.01% (w/w) Cd. Characteristic of Zn hyperaccumulators are dramatically elevated rates of root-to-shoot translocation of Zn and very high tolerance to Zn, especially in above-ground tissues. However, hyperaccumulators are generally low-biomass plants and unsuitable for large-scale phytoremediation. Understanding the molecular basis of

Zn and Cd hyperaccumulation and associated hypertolerance may help to develop effective phytoremediation technologies [30,29].

## 3. How do plants maintain Zn homeostasis?

In order to maintain Zn concentrations within physiological limits, land plants modulate the processes contributing to Zn homeostasis throughout the plant according to external supply and internal requirements [15,31]. This begins with the acquisition and uptake of Zn from the soil in roots. Zn can bind tightly to soil and plant cell wall components, and it can form precipitates, most commonly in the form of phosphates, carbonates or hydroxides, in the soil. As is also the case for Fe(III), which exhibits even lower solubility, Zn solubilisation in the rhizosphere is thought to occur *via* plant-mediated acidification and secretion of low-molecular-weight organic chelators. However, their specific roles and contributions to plant Zn acquisition remain poorly understood. Subsequently, Zn is taken up across the plasma membrane of root cells predominantly as a free ion (Fig. 1). Root uptake of Zn-phytosiderophore complexes, which are formed with phytosiderophores that are primarily known as Fe(III) chelators secreted by plant roots, has been reported in grasses [32] (see below).

In the cytoplasm of plant cells, Zn is thought to be chelated by low-molecular-weight ligands in order to prevent cytoplasmic precipitation and non-specific binding to biomolecules. The roles of the rather abundant plant metallothionein proteins, which have only limited sequence similarity to those of animals or yeast, remain poorly understood [33]. A subset of plant metallothioneins is likely to contribute to the buffering or storage of cytosolic Zn rather than act as Zn-trafficking metallochaperone proteins [34], which were postulated earlier based on findings concerning Cu homeostasis. Once in the root symplast, Zn can be immobilized in the root through transport into vacuoles, or it can undergo symplastic transport, which is thought to occur via plasmodesmata, towards and into the vascular cylinder (Fig. 1). Recent work suggests that the speciation of Zn in the root symplasm may influence the extent of Zn immobilization in root vacuoles [35–37]. Moreover, recently published work suggests that the export of Zn across the inner plasma membrane of root epidermal cells promotes radial transport of Zn towards the stele of the root and has an important role under Zn deficiency [38]. In Arabidopsis and almost all other plants, root vacuoles are a major storage site for excess Zn, and the Zn thus removed from the root-to-shoot transport pathway makes an important contribution to basal Zn tolerance [25].

The export from cells is required for the loading of Zn into the apoplastic xylem and thus for the translocation of Zn from the root to the shoot [26,39] (Fig. 1). Inside the xylem, Zn flux into the shoot is mass-flow driven. There is some evidence for the chelation of Zn by low-molecular-weight ligands inside the xylem, which could act to prevent Zn retention by metal-binding components of the surrounding cell walls or uptake into cells *via* $Zn^{2+}$ transporters. Inside the shoot, Zn must be taken up from the xylem across the plasma membrane of adjacent cells (Fig. 2). Little is known about how Zn is distributed between cells. Generally, trichomes and epidermal cells, in particular, accumulate the highest Zn concentrations [31] (Fig. 2). There is some evidence for the extracellular accumulation of Zn in trichomes [40]. However, cell vacuoles are believed to make the largest contribution to the storage of excess Zn in leaves. The remobilization of Zn, in particular in photosynthetic source tissues and in senescing leaves, for the translocation into sink tissues, i.e., meristems, developing leaves, inflorescences and developing seeds, *via* long-distance, mass flow-driven transport in the phloem is common [41] (Fig. 2). In contrast to the xylem, the phloem transport pathway consists of strongly modified living cells that are symplastically connected among each other and with the adjacent companion cells [42]. Because of the high concentrations of off-target metal-binding compounds and the higher pH inside the phloem, the chelation of Zn is particularly important for long-distance transport inside the phloem. The transport of compounds into companion cells for subsequent source-to-sink translocation *via* the phloem occurs either symplastically or from the apoplast through cellular uptake, depending on the plant species [43]. The close proximity of xylem and phloem indicate the possibility of an exchange of solutes between these two long-distance transport pathways, most likely at the nodes where leaves or side branches emerge.

### 3.1. Membrane transporters and metal-binding compounds acting in Zn homeostasis

In plants, Zn-dependent processes are located in all cellular compartments, including cytoplasm, mitochondria [44] and chloroplasts [45], as well as the nucleus [46] (reviewed in [47]). Cell vacuoles are the major site for storage and detoxification of excess Zn and a source for Zn remobilisation in periods of deficiency. $Zn^{2+}$ cations are strong Lewis acids and readily form complexes with numerous organic molecules. In order to prevent uncontrolled binding of $Zn^{2+}$ to non-target sites, it is believed that Zn is largely present in bound form in the cytoplasm and in other cellular compartments. In contrast to Cu, no metallochaperone proteins that act in the trafficking of Zn to specific target apo-metalloproteins have been identified in plants to date. Three proteins homologous to bacterial COG0523-domain proteins, which were proposed to function in the maturation of metalloproteins, are encoded in the genome of *A. thaliana*, but none of these have been functionally characterized to date [48]. Instead, there is more and more evidence to suggest that low-molecular-weight metal chelators might be important ligands for Zn in plants. Using comparative amino acid analysis and Extended X-ray Absorption Fine Structure (EXAFS) analysis, the free amino acid histidine was initially implicated in nickel chelation and shown to be of particular importance in Ni hyperaccumulation and hypertolerance [49], and it was later also implicated in the binding of cytoplasmic Zn in a Zn hyperaccumulator [50]. Nicotianamine (NA), a non-proteinogenic amino acid acting as high-affinity metal chelator, was discovered through a comparative bioanalytical approach utilizing a deficient tomato mutant [51]. NA, first proposed to act in Fe and Cu homeostasis, appears to also have a central role in Zn homeostasis of plants and filamentous fungi [52,53,35,36], ensuring symplastic cell-to-cell and phloem mobility of Zn. In addition, there is some evidence that the thiols glutathione (γ-ECG) and phytochelatins ($(\gamma\text{-EC})_n$G, $n = 2$ to 9) contribute to basal Zn tolerance of Arabidopsis [54,55]. To date, no contribution has been demonstrated of phytochelatins or glutathione to naturally selected metal hypertolerance. Further compounds that have been proposed to bind Zn in plants are organic acids, in particular malate, citrate and oxalate, phosphate, phytate and pectates [56–58], reviewed in [59,60]. It is not known to which extent Zn homeostasis is affected by a reduction in the capacity for Zn binding through these compounds.

Some plant membrane transporters that contribute to plant Zn homeostasis transport $Zn^{2+}$ cations [61], whereas others transport a Zn-ligand complex [32,62]. Finally, it appears that an additional, major contribution to plant Zn homeostasis can be made by membrane transporters of ligands alone, whereby ligands may act subsequent to their transport by binding $Zn^{2+}$ on the opposite side of the membrane [36].

The possible involvement of Zinc-Regulated Transporter, Iron-Regulated Transporter (ZRT-IRT)-like proteins (ZIPs) in cellular $Zn^{2+}$ uptake was established by expressing cDNAs from Zn-deficient plants in a yeast *zrt1zrt2* mutant [63]. Some plant members of the so-called Cation Diffusion Facilitator (CDF) family of metal cation/proton antiporters, members of which have also been named ZAT (Zinc Transporter of Arabidopsis thaliana) and MTP (Metal Tolerance Protein or Metal Transport Protein), act in the removal of Zn from the cytoplasm. The first plant MTP was identified serendipitously upon overexpression of a cDNA in Arabidopsis and by the similarity of the encoded protein to human ZnTs [64]. Reverse genetics have

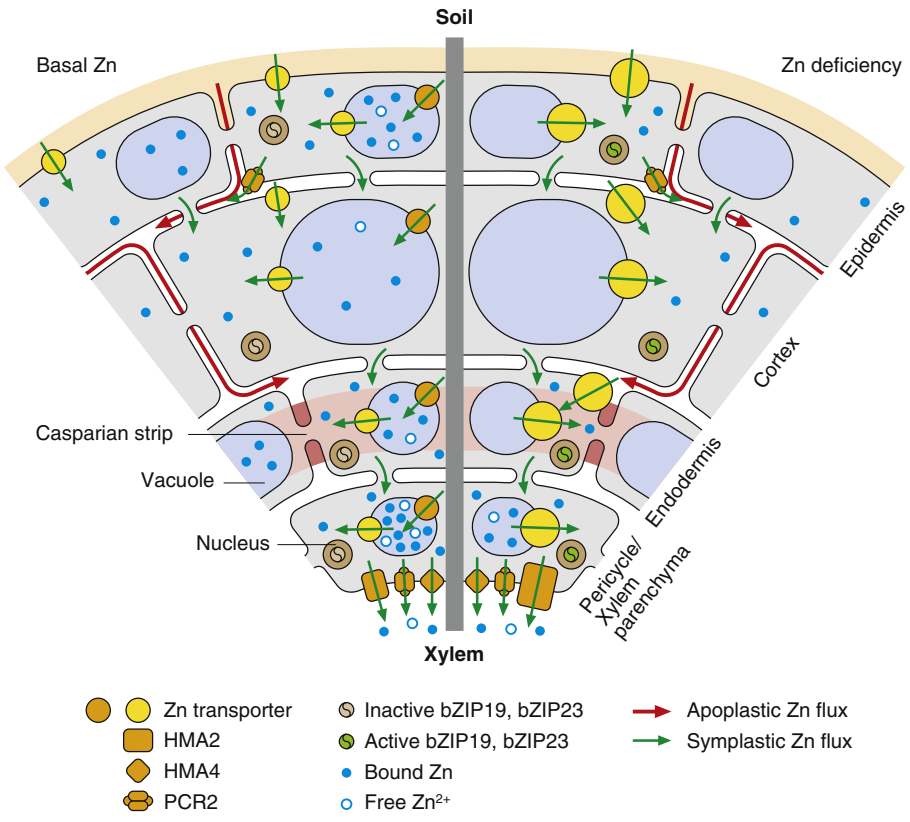


**Fig. 1.** Root responses to Zn deficiency. Shown are sectors of transverse sections of a Zn-sufficient (left) and a Zn-deficient (right) root, together with Zn movement pathways, initiating in the soil solution on the external surface of the root, and ending in the apoplastic xylem vessels, in which Zn and other nutrients move to the shoot with the transpiration stream. Sizes of transporter symbols represent protein levels extrapolated from transcript abundance, and the number of Zn symbols approximates Zn concentrations in different locations inside the root. Zn deficiency responses include increased uptake into the root symplast, enhanced re-mobilization of Zn from the vacuoles (postulated) and increased xylem loading. According to the current working model, the active bZIP19/bZIP23 complex enhances transcription of *ZIP1*, *ZIP3*, *ZIP4*, *ZIP5*, *ZIP9*, *ZIP10*, *ZIP12*, *IRT3*, *NAS2* and *NAS4* [89]. *HMA2* transcript levels are also known to increase under Zn deficiency [90], but the underlying mechanism remains unknown. The representation of Zn concentrations in the root is based on Zinpyr-1 fluorescence imaging [38,39]. The shown model is based on *Arabidopsis thaliana*.

been used extensively in the identification of membrane transporters contributing to Zn homeostasis. Plant members of the Natural Resistance-Associated Macrophage Protein (NRAMP) family acting in proton-driven transition metal cation transport were identified by sequence similarity to NRAMPs of other organisms [65,66]. The YSL (Yellow Stripe-Like) family in Arabidopsis was named based on

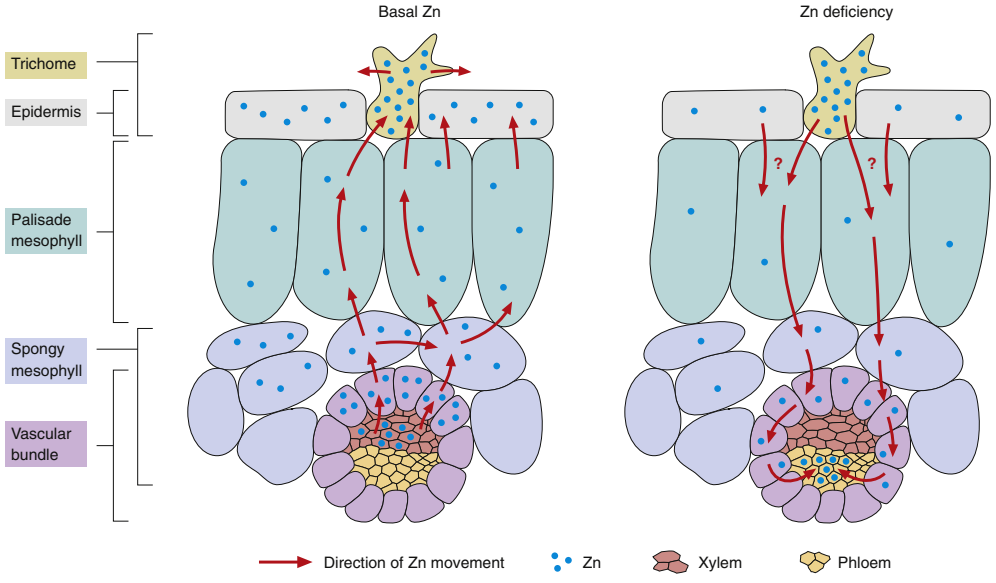


**Fig. 2.** Shoot responses to Zn deficiency. Shown are the upper parts of transverse sections of a Zn-sufficient leaf (left) and a Zn-deficient leaf (right), with hypothetical pathways of Zn movement. The number of Zn symbols represents Zn concentrations. Zn accumulation in trichomes has been shown in several species including Zn hyperaccumulators and non-accumulators [40,58]. It is thought that in Zn-deficient plants and in senescent leaves, stored Zn can be remobilized *via* the phloem. The shown model summarizes findings in various Brassicaceae.

the sequence similarity of these transporters to the *Zea mays* YS1 (Yellow Stripe 1) transporter [67]. Finally, Arabidopsis HMA1 to 4 proteins (Heavy Metal ATPases of the $P_{1B}$-type ATPases) share considerable similarity with bacterial divalent transition metal cation pumps [68]. Heterologous screening in yeast led to the identification of PCR (Plant Cadmium Resistance) proteins acting in cellular export of various divalent metal cations [69]. One member of this family of membrane transport proteins, PCR2, was later shown to contribute to Zn homeostasis using reverse genetics [38]. A Major Facilitator Superfamily (MFS) transporter, Zinc-Induced Facilitator 1 (ZIF1), was identified to contribute to basal Zn tolerance in Arabidopsis through a classical forward genetic screen [53].

### 3.2. $Zn^{2+}$ import into the cytoplasm by ZIP family transporters

In yeast, ZRT1 and ZRT2 are ZIP transporters responsible for the acquisition of Zn, and growth impairment of a *zrt1zrt2* mutant can be restored by augmenting external Zn supply [46]. In humans, a number of ZIP transporters have been implicated in transporting metals into cells, for example ZIP4, which is important in the uptake of Zn from the gut [70], and ZIP10, which is involved in renal Zn re-absorption [21].

The genome of Arabidopsis encodes 15 ZIP transporters [71], the best characterised being IRT1 (Iron-Regulated Transporter 1). IRT1 is the main transporter responsible for root uptake of $Fe^{2+}$ from the soil solution in epidermal cells of the root hair zone of non-graminaceous plants [72]. In accordance with this, *irt1* mutants are severely Fe-deficient and die at the seedling stage unless rescued by exogenous application of Fe-chelates [73,74]. As Fe is present in the oxidized and insoluble Fe(III) form in most soils, the action of Ferric Reductase Oxidase2 (FRO2), which reduces Fe(III) to the more soluble $Fe^{2+}$ on the external side of the plasma membrane [75], is required prior to the uptake of $Fe^{2+}$ into the cell *via* IRT1. Both FRO2 and IRT1 protein levels are much higher in Fe-deficient plants than in Fe-replete plants, and are regulated both transcriptionally and post-translationally [76–78]. This is similar to the regulation of ZRT2 in yeast, which undergoes ubiquitin-mediated degradation in Zn-replete conditions [79]. Split-root experiments showed that transcript levels of *IRT1* and *FRO2* are regulated by a combination of both local root and shoot-derived long-distance signals. In Fe-replete conditions *IRT1* and *FRO2* transcripts also accumulate specifically during the day, suggesting the possibility of circadian regulation of Fe acquisition [80,59]. *IRT1* and *FRO2* transcript levels are upregulated not only in response to iron deficiency, but also in the presence of excess Zn in the growth medium [81]. In agreement with this, growth under excess Zn conditions led to increased root surface ferric chelate reductase activity [81] and IRT1 protein levels [82], although the latter result is not unequivocal [76] (see also below). In addition to transporting $Fe^{2+}$, IRT1 non-selectively mediates the uptake of several divalent transition metal cations into the root symplasm, thus acting as a major pathway for the influx of $Zn^{2+}$ and $Cd^{2+}$ [72]. Transcript levels of a number of genes encoding proteins that contribute to Zn and Cd detoxification [83,25,36] are upregulated under Fe deficiency when IRT1 protein levels are strongly increased (Fig. 3, see also below).

IRT2 is a close homologue of IRT1 and thought to have a similar, albeit less important, role. *IRT2* is expressed in the external cell layers of the root sub-apical zone, and *IRT2* expression in yeast can rescue mutants defective in cellular Fe and Zn uptake [84]. The IRT2 protein was localised to intracellular vesicles, and *IRT2* transcript levels are tightly co-regulated with those of *IRT1* and *FRO2*. This is consistent with a role for *IRT2* in maintaining cellular Fe homeostasis [85].

The only additional well-characterised ZIP from Arabidopsis is IRT3. *IRT3* transcript levels are strongly upregulated in response to Zn deficiency and are constitutively high in roots of the Zn hyperaccumulators *A. halleri* and *N. caerulescens* [81,86,87]. IRT3 can transport Zn and Fe when expressed in yeast, confers increased shoot Zn and Fe accumulation when over-expressed in transgenic plants, and AtIRT3 and AhIRT3 were localized to the plasma membrane [88]. Thus, IRT3 was proposed to be capable of transporting both $Zn^{2+}$ and $Fe^{2+}$ across the plasma membrane into the cell.

Little is known about the roles of other Arabidopsis ZIP proteins *in planta*. Expression of Arabidopsis *ZIP1*, *ZIP2*, *ZIP3* and *ZIP4* cDNAs can complement the *zrt1zrt2* double mutant of yeast, showing that they are all capable of mediating cellular Zn uptake across the plasma membrane [63,89]. Expression of *ZIP4* also complemented the yeast *ctr1* mutant defective in cellular Cu(I) uptake, suggesting that it can also transport Cu in a heterologous system [90]. In Arabidopsis, transcript levels of 10 ZIP family genes increase in response to Zn deficiency [30] (see Fig. 1). Comparisons between Zn/Cd hyperaccumulators *A. halleri* or *N. caerulescens* and related non-accumulators have implicated increased expression of *ZIP4*, *ZIP6*, *ZIP9*, *ZIP10* and *IRT3* in Zn/Cd hyperaccumulation [30]. Although there is much circumstantial evidence for the involvement of ZIP proteins in Zn homeostasis, the scarcity of functional data makes it difficult to assign specific physiological roles.

### 3.3. $Zn^{2+}$ export from the cytoplasm by a subgroup of HMA proteins of the $P_{1B}$-type ATPase family

Of the eight members of the $P_{1B}$-type ATPase protein family in Arabidopsis, HMA5, HMA6, HMA7 and HMA8 are homologous to prokaryotic $Cu^{+}$/$Ag^{+}$-transporters [68] and do not contribute to $Zn^{2+}$ transport. The function of HMA1 remains unclear. Bacterial homologues were reported to transport divalent transition metal cations. A chimeric GFP fusion protein was localised to the chloroplast envelope. Different reports suggested that it is a Cu transporter [91], a Zn transporter [92] or a Ca transporter [93].

HMA2, HMA3 and HMA4 form a monophyletic group sharing significant sequence similarity and are most closely related to prokaryotic $Zn^{2+}$/$Cd^{2+}$ pumps [68]. The Columbia (Col-0) accession of Arabidopsis exhibits a single base-pair deletion in *HMA3* that produces a frame-shift and a premature stop codon [26], thus representing a natural loss-of-function allele of *HMA3*. GFP-tagged functional HMA3 was reported to localise to the vacuolar membrane in both yeast and Arabidopsis [94,95]. In the Wassilewskaja (Ws) accession of *A. thaliana*, in which the *HMA3* locus encodes an intact, full-length protein, an *hma3* mutant is more sensitive than WT to Cd and Zn, and overexpression of *HMA3* increased Zn and Cd tolerance and enhanced Cd accumulation in both shoots and roots [95]. *HMA3* transcript levels are elevated in shoots of metal hyperaccumulators [81,96,97], suggesting that HMA3 could contribute to their Zn and Cd hypertolerance by moving these metals into the vacuole. *HMA3* is among the genes with a likely role in Zn detoxification, for which root transcript levels increase under Fe deficiency [84] (see above and Fig. 3).

Initially, *hma2* or *hma4* single mutants were reported not to have visible growth phenotypes and not to be hypersensitive to Zn or Cd [26,98,99,18]. In a later study, however, two allelic *hma4* mutants in the Columbia (Col-0) genetic background were found to be more sensitive to Cd and Zn than wild-type seedlings [101]. In addition, *hma4* single mutants accumulate more Zn in roots and less in shoots than the wild type [26,18]. Arabidopsis *hma2hma4* double mutants are Zn deficient in the shoots, resulting in chlorosis, stunting of growth, increased shoot branching and infertility, whereas root-to-shoot partitioning of other essential transition metals is unaffected. Zn deficiency of the shoot can be rescued by the application of exogenous Zn [26]. The Zn accumulated in the roots of the *hma4* and *hma2hma4* mutants can be visualised predominantly inside root pericycle cells using the Zn fluorophore Zinpyr-1 [18]. In roots, lines transformed with *HMA2promoter-GUS* and *HMA4promoter-GUS* fusion constructs show reporter activity in the vasculature [26]. Using a *HMA2promoter-HMA2cDNA-GFP* fusion construct encoding a chimeric HMA2-GFP fusion protein [18], HMA2 was localized in the plasma

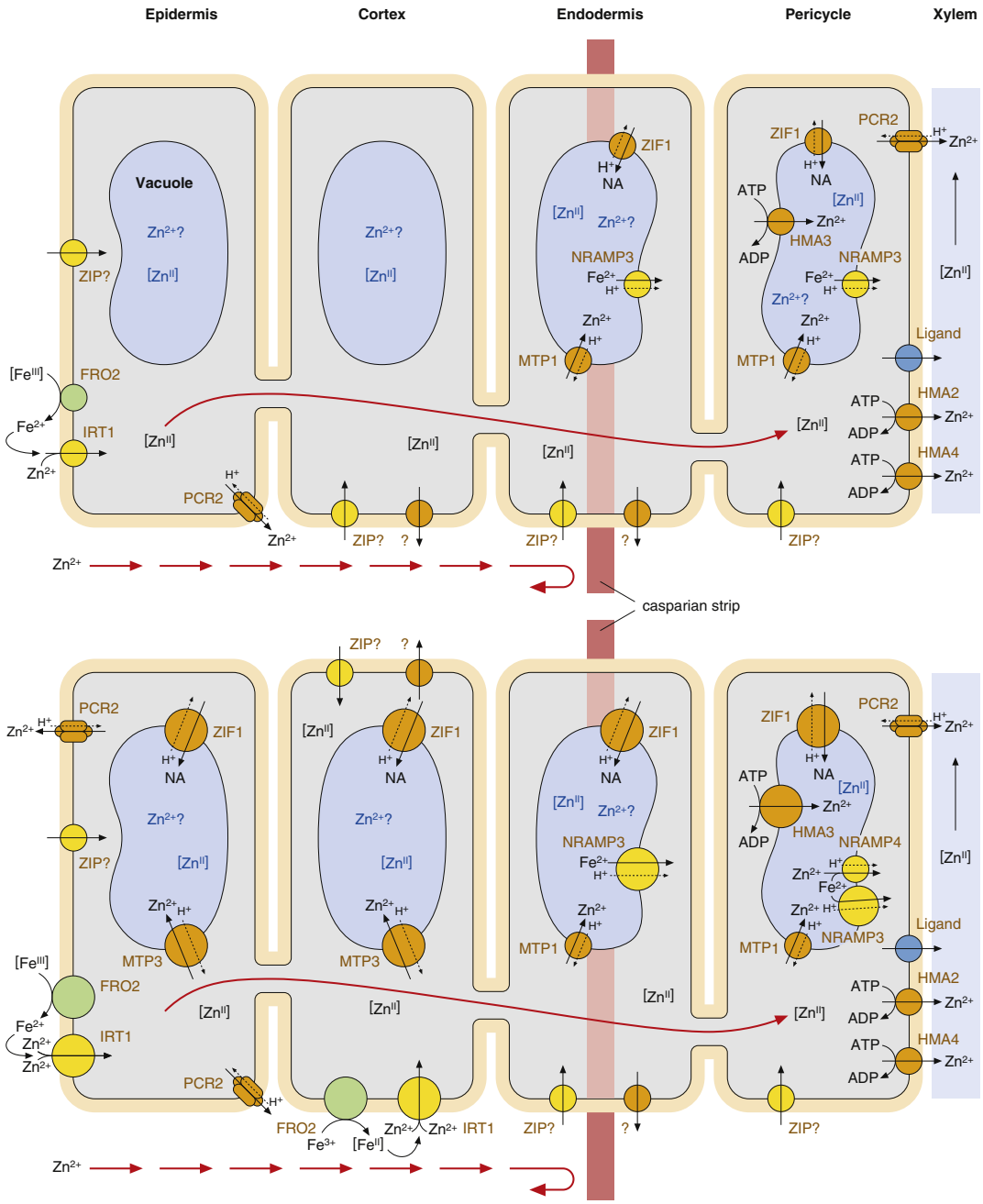


**Fig. 3.** The interdependence of Zn and Fe homeostasis. Shown are membrane transport processes contributing to Zn and Fe homeostasis across root cell layers of Fe-sufficient (upper panel) and Fe-deficient (lower panel) plants in a longitudinally sectioning view. Sizes of transporter symbols represent protein levels extrapolated from transcript abundance, partially supported by proteomics data (see main manuscript text). The major root epidermal plasma membrane Fe transporter IRT1 mediates the uptake of $Zn^{2+}$ as well as its primary substrate $Fe^{2+}$ [72]. Thus, when IRT1 protein levels increase under Fe deficiency and likely also under excess Zn, the plant has to accommodate an enhanced influx of Zn. This involves MTP3 [25] and ZIF1 [36,136], among others (see main manuscript text). The shown model is based on *Arabidopsis thaliana*.

membrane of pericycle cells. GUS reporter lines indicated that *HMA4* is expressed in pericycle and xylem parenchyma cells of *A. thaliana* and *A. halleri*, and this was confirmed in *A. halleri* by *in situ* hybridization [39]. Several studies have reported HMA2 [26] and HMA4 [99] localization in the plasma membrane of plant and yeast cells. When expressed in *S. cerevisiae*, *HMA4* cDNAs complement Zn- and Cd-hypersensitive mutants and decrease cellular metal accumulation rates [100,110]. All available evidence is thus consistent with a role for both *HMA2* and *HMA4* in the loading of Zn into the xylem by pumping Zn out of the adjacent cells in the root. *HMA2* and *HMA4* are also the major contributors to root-to-shoot translocation of Cd [102]. However, the biological roles of *HMA2* and *HMA4* are not fully identical because only *HMA2* transcript levels increase in response to Zn deficiency [90] (Ina Talke and Ute Krämer, unpublished data).

In the Zn/Cd hyperaccumulator *A. halleri*, *HMA4* transcripts are substantially more abundant than in *A. thaliana* [86]. This is caused by a triplication of the gene in the genome, as well as strongly activating mutations in the promoters of all three gene copies of *A. halleri* [39]. Using an RNAi approach, *HMA4* was found to be necessary for the hyperaccumulation of Zn in *A. halleri*, i.e., for the accumulation of Zn to very high levels specifically in the shoot. Moreover, Cd hypertolerance and – to a lesser extent – Zn hypertolerance of *A. halleri* were shown to be partially dependent on *HMA4*. Expression of an *AhHMA4promoter-AhHMA4* cDNA fusion

construct in *A. thaliana* conferred increased Zn translocation into the xylem, but was not sufficient for increasing plant Zn or Cd tolerance, which is thought to additionally require enhanced expression of other genes [39,29]. Transcripts of *HMA4* were also detected in the cambium and – at lower signal intensities – in the xylem parenchyma of leaf veins [39]. The functional significance of this observation remains to be established. A tandem quadruplication of the *HMA4* locus was also identified in *N. caerulescens* and hypothesised to contribute to the Zn/Cd hyperaccumulation trait characteristic of this species [103].

### 3.4. $Zn^{2+}$ export from the cytoplasm by some Arabidopsis MTPs of the Cation Diffusion Facilitator (CDF) family

The 12 Arabidopsis MTPs belong to several distinct phylogenetic groups likely to differ in substrate specificity [104,105]. Arabidopsis MTP1 and MTP3 are the well-characterized Zn-transporting MTP proteins in a common phylogenetic group. Both *MTP1* and *MTP3* cDNAs are able to complement the Zn-hypersensitive yeast *zrc1cot1* double mutant, and the proteins localize to the vacuolar membrane, suggesting that they can transport Zn into the vacuole [106,107,25]. However, *MTP1* and *MTP3* appear to have distinct, only partially overlapping expression patterns in leaves and roots based on the analysis of transgenic plants carrying promoter-reporter gene fusion constructs. *MTP1* promoter activity is highest in young leaves, whereas *MTP3* expression is undetectable in shoots [107,25]. In roots, *MTP1* promoter activity is very high in the root tip including the meristematic and elongation zones. *MTP1* promoter activity is also high in the vasculature of roots of young seedlings, but decreases with increasing seedling age [107]. By contrast, *MTP3* expression is very low under normal growth conditions and strongly increased in response to Fe deficiency and upon exposure of seedlings to excess Zn, specifically in epidermal and cortex cells of the root hair zone [25]. Arabidopsis genotypes with reduced *MTP1* or *MTP3* functions are hypersensitive to Zn [106,107,25]. However, *MTP1* and *MTP3* have opposite effects on Zn accumulation. Whereas *MTP3* acts to decrease shoot Zn concentrations, *MTP1* increases total Zn concentrations in leaves [107,25]. The main role of *MTP3* appears to be the removal Zn from the root-to-shoot transport pathway under conditions of high Zn influx into roots [25]. By contrast, *MTP1* appears to function in Zn sequestration in sensitive dividing and expanding tissues and may operate to generate Zn stores or to direct Zn accumulation in specific tissues of the shoot [108]. *MTP1* orthologues are highly expressed in metal hyperaccumulator species such as *A. halleri*, *N. goesingense* and *N. caerulescens*, and their involvement in Zn hypertolerance has been proposed [109,81,110,108,111]. Biochemical analyses of *A. thaliana* MTP1 established that the His-rich cytoplasmic loop is not essential for Zn transport activity, but could function to buffer cytoplasmic Zn, and may act as a sensor of cytoplasmic Zn levels. Deletion of this loop resulted in a hyper-active MTP1 protein [112]. Recent work building on this used random and site-directed mutagenesis to reveal important residues for metal selectivity in the His-rich intracellular loop and the third transmembrane helix of the protein [113].

Four of the Arabidopsis MTPs are homologous to MTP8 of the legume *Stylosanthes hamata*, which acts in the transport of $Mn^{2+}$ into the vacuole [114]. The best characterised member of this phylogenetic group in Arabidopsis is MTP11, which transports Mn when expressed in yeast and was functionally characterized in detail [104,115].

### 3.5. $Zn^{2+}$ transport through some NRAMP proteins

NRAMP proteins are transition metal cation/proton co-transporters or anti-porters of generally broad specificity [116,117]. Recent work on *A. thaliana* NRAMP1 has shown that this transporter is the major plasma membrane Mn uptake system in roots. *NRAMP1* transcript levels increase under Mn deficiency, and accordingly, *nramp1* mutants are sensitive to Mn deficiency [118]. Expression of *A. thaliana NRAMP1*, *NRAMP3* and *NRAMP4* cDNAs in yeast rescued Fe- and Mn-uptake defective mutants [119,66], whereas only *NRAMP4* appeared to confer Zn uptake. In *A. thaliana* both NRAMP3 and NRAMP4 localize to the vacuolar membrane [120]. Both proteins have overlapping functions in mobilising Fe from vacuolar storage during seed germination. Consequently, *nramp3nramp4* double mutant seeds were unable to complete seedling development on media low in available Fe [121]. In vegetative plants, however, these two proteins are more important in the remobilization of vacuolar Mn, providing evidence for an important role of the leaf vacuole as a site for transitory Mn storage and consistent with an only minor role of the vacuole in leaf Fe storage [122]. The Zn and Cd hypersensitivity of *nramp3nramp4* double mutants [123] is likely to be an indirect effect, primarily resulting from impaired remobilization of Fe and Mn from the vacuole. It appears that other transporters, likely of the ZIP family, are more important in the remobilization of Zn from the vacuole. In hyperaccumulators, transcript levels of orthologues of *NRAMP3* and *NRAMP4* are much higher than in closely related non-accumulators, but the biological significance of this observation remains to be elucidated [123,124].

### 3.6. Import of Zn-ligand complexes into the cytoplasm through some YSL proteins

The first member identified of the Yellow Stripe-Like (YSL) transporter family in the Oligopeptide Transporter (OPT) superfamily, Yellow Stripe 1 (YS1), is of central importance in Fe acquisition in *Zea mays*, mediating the uptake of Fe(III)-phytosiderophore complexes into root epidermal cells [67]. Orthologues in other grasses, HvYS1 and OsYSL15, have analogous functions [125,126]. Subsequent work has shown that YSL proteins transport complexes of various transition metals, with either phytosiderophores or nicotianamine (NA) acting as the ligand, into the cytosol by proton-coupled symport [62,128]. Since dicotyledonous plants do not synthesise phytosiderophores, the eight members of the Arabidopsis YSL family are expected to transport metal–NA complexes [62]. Upon expression of *AtYSL2* in yeast, cellular uptake was reported of Fe(II)–NA and Cu(II)–NA, and *AtYSL2* transcript abundance was found to be downregulated in response to Fe deficiency and Cu excess [128]. Using *YSL2promoter-GUS* lines, promoter activity was detected in many cell types, and was particularly strong in cells around the vasculature, suggesting that *YSL2* functions in the lateral movement of metals from the vasculature [128]. A later study was unable to find evidence for Fe(II)–NA or Fe(III)–NA transport into yeast cells expressing *AtYSL2* [129]. Furthermore, *YSL2* transcript levels were found to be downregulated under both Fe and Zn deficiency.

A *ysl1* mutant of *A. thaliana* exhibited reduced levels of NA in leaves, a decrease in both NA and Fe concentrations in seeds and a slower seed germination that could be rescued by supplying Fe [130]. Arabidopsis *ysl1ysl3* double mutants exhibited Fe deficiency symptoms including inter-veinal chlorosis [131]. This was associated with reduced Fe and increased Cu, Zn and Mn levels in leaves. After observing that expression of *YSL1* and *YSL3* was highest in senescing leaves, and that seeds of *ysl1ysl3* were Fe-, Zn- and Cu-deficient, it was proposed that *YSL1* and *YSL3* are important in remobilising metals from senescing tissues for use in reproductive organs.

Among the eight Arabidopsis YSLs, only one is known to be strongly regulated depending on plant Zn status. Transcript levels of *YSL2* are repressed under Zn deficiency [128,129]. By contrast, transcript levels of *NAS2* encoding one of four isoforms of NA Synthase (NAS) in Arabidopsis are strongly increased under Zn deficiency (see below). It should be taken into account that, even in the absence of Zn-dependent transcriptional regulation, the rate of Zn transport through YSL proteins can be dependent on the local concentration of the Zn-NA complex that may increase when NA levels generally

increase, i.e., presumably under Zn deficiency, under Zn excess and Fe deficiency [36]. Alternatively, it is also possible that the Zn-dependent transcriptional regulation of a YSL transporter not contributing to Zn transport could result from an indirect effect, for example from a secondary alteration in internal Fe or Cu status. Further direct evidence for the substrates transported by YSL transporters is required in order to improve our understanding of their functions.

### 3.7. Export of metal complexes from the cytoplasm through some ATP-Binding Cassette (ABC) transporters

There are 132 loci in Arabidopsis that encode ABC transporters [132,133], a number of which have been implicated in cellular Cd detoxification. In *A. thaliana* the transporters ABCC1 and ABCC2 were shown to contribute to the accumulation of Cd–phytochelatin (Cd–PC) complexes in the vacuole [134]. The chemical similarity of $Cd^{2+}$ to $Zn^{2+}$ and the finding that PCs are also important in Zn homeostasis [54] suggest the possibility that these transporters or their homologues might contribute to Zn homeostasis as well as Cd tolerance.

### 3.8. Export of Zn ligands from the cytoplasm through members of the ZIFL family of Major Facilitator Superfamily transporters

The Arabidopsis *ZIF1* gene was discovered through a genetic screen for Zn-hypersensitive mutants [53]. *ZIF1* encodes a predicted proton-organic substrate antiporter and was the founding member of the ZIF1-like (ZIFL) protein family of Major Facilitator Superfamily (MFS) transporters [135]. *ZIF1* is expressed in dividing and expanding cells and in the vasculature of mature tissues. Expression is increased and expands to a much broader range of cell types upon exposure to high Zn and Fe deficiency [136,137,36]. The ZIF1 protein localizes to the vacuolar membrane [53]. It was speculated that, due to pH constraints, most Zn is chelated by organic acids in plant vacuoles and that ZIF1 may transport an organic acid or a Zn–organic acid complex into the vacuole [53]. However, recent work implicates ZIF1 in the transport of NA into the vacuole [36]. Interestingly, enhancing vacuolar accumulation of NA through overexpression of *ZIF1* leads to strongly enhanced vacuolar Zn accumulation. As a consequence, Zn is immobilized in roots and root-to-shoot transport of Zn is decreased in *ZIF1* overexpressors, whereas root-to-shoot transport of Fe is normal or even slightly enhanced. *ZIF1* overexpressors show NA-deficiency as well as Zn- and Fe-deficiency symptoms indicating a lack of cytosolic NA, which can be corrected by spraying with Zn and Fe. Under Fe deficiency, *ZIF1* contributes to Zn detoxification similar to *MTP3* and *HMA3* (Fig. 3). However, *ZIF1* appears to have an additional, direct role under Fe deficiency that remains to be fully elucidated [36]. Interestingly, the transcriptional regulator POPEYE (PYE), a bHLH protein, was reported to directly interact with the *ZIF1* promoter and to repress its transcription, although there is a concurrent net increase in *ZIF1* transcript levels, under Fe deficiency [136]. Tightly balanced vacuolar NA compartmentalization through ZIF1 is thus critical for the maintenance of Fe homeostasis [136,36]. Additionally, this recent work indicates that vacuolar NA compartmentalization is important in the differentiation between Fe and Zn inside the plant, subsequent to their largely non-specific uptake. The only other ZIFL protein characterized to date is *Oryza sativa* TOM1. OsTOM1 was proposed to act in phytosiderophore secretion from rice roots integral to the acquisition of Fe, and likely also Zn, in graminaceous plants [138].

### 3.9. Export of $Zn^{2+}$ from the cytoplasm through PCR2

Recently, Arabidopsis PCR2, a cysteine-rich protein with only two transmembrane helices localizing to the plasma membrane was shown to be capable of mediating the export of Zn and Cd from yeast cells, acting as a homo-multimer [38]. *PCR2* is expressed in vascular tissue of shoots, and strongly in the epidermis of root cells, in the xylem of the root elongation zone and in the root tip. Arabidopsis *pcr2* mutants accumulate Zn and Fe in roots implying a role in root-to-shoot translocation of metals, independent of *HMA2* and *HMA4*. Interestingly, *pcr2* single mutants were sensitive both to an excess and a deficiency of Zn. Along with the expression pattern, the currently available data suggest that *PCR2* has dual roles in Zn homeostasis by acting in the detoxification of excess Zn and in nutrient use efficiency of Zn [38].

### 3.10. Low-molecular-weight Zn ligands and Zn-binding proteins

The strong phenotype of the *zif1* mutant illustrates the importance of ligands acting in Zn homeostasis [53,36]. By forming complexes with $Zn^{2+}$, ligands can function to sequester, detoxify or store high levels of metals or act as buffers or chaperones for the movement of metals within the plant. Ligands may be secreted from cells to enhance the solubility or mobility of an essential metal [139]. According to the present state of knowledge, the most important metal-binding low-molecular-weight ligands are nicotianamine, in grasses phytosiderophores, and histidine, phytochelatins and organic acids. Furthermore, metal-binding proteins may make a major contribution to Zn homeostasis, but their role remains poorly understood.

### 3.11. Phytosiderophores and nicotianamine (NA)

Phytosiderophores, such as mugineic acid, are important in the acquisition of Fe, and also contribute to Zn acquisition [32], by graminaceous plants. In these plants, phytosiderophores are enzymatically synthesized from *S*-adenosyl methionine *via* the intermediate NA, and are secreted into the rhizosphere. Fe(III)-phytosiderophore complexes are taken up into the root by members of the YSL family of transporters [67]. This can be verified by complementing yeast mutants defective in Fe uptake [128,129] in the presence of phytosiderophores added to the growth medium. Genes of the phytosiderophore biosynthetic pathway are induced in Zn- as well as Fe-deficient barley, resulting in increased secretion of phytosiderophores, further supporting that phytosiderophores are also involved in Zn acquisition [62,140].

Non-graminaceous plants do not produce phytosiderophores, but synthesise the precursor NA, which was shown to be involved in intracellular metal chelation, and in inter-cellular and long-distance phloem metal transport [141]. In the *chloronerva* mutant of tomato, the single *NAS* gene is mutated, resulting in inter-veinal chlorosis of young leaves, reduced growth and sterility, a phenotype resembling that of Fe-deficiency, irrespective of external Fe supply [142]. The mutant is rescued by supplying exogenous NA, suggesting that phloem Fe transport and the movement of Fe out of the shoot vasculature are defective in the *chloronerva* mutant [143,144]. In *A. thaliana* carrying mutations in all four *NAS* genes, the most evident defect is an impaired ability to move Fe to developing seeds [35].

In *A. thaliana, NAS* transcript levels are upregulated in Fe-, Zn- and Cu-deficient plants [90]. A thorough physiological analysis of transgenic tobacco plants containing decreased NA levels demonstrated that NA is functionally involved not only in long-distance transport of Fe, but also in long-distance transport and inter-cellular movement of Cu and Zn [144,127]. Compared to *A. thaliana*, substantially elevated root *NAS2* transcript levels contribute to Zn hyperaccumulation of *A. halleri* [96,86,145,37]. Heterologous expression of *A. halleri NAS* cDNAs in yeast conferred increased Zn tolerance [96,81]. At low pH values, the stability of metal-NA complexes is lower than that of metal-phytosiderophore complexes. Therefore, NA is expected to act primarily in the cytoplasm and in the phloem. However, the formation of stable Zn-NA complexes at vacuolar pH values is possible [146,147]. Moreover, in transgenic plants the enhanced

compartmentalization of NA in the vacuole directed the vacuolar accumulation of Zn [36].

### 3.12. Histidine and organic acids

Free histidine has a high affinity for the binding of transition metal cations, yet much lower than that of NA, and it is important in Ni hyperaccumulation [49,148]. X-ray absorption fine structure studies in the Zn hyperaccumulator *N. caerulescens* have shown Zn-histidine to be the second most abundant Zn-ligand species [50]. It is speculated that Zn-histidine complexes are formed at the typical pH values found in the cytosol, similar to NA complexes [148,149]. By comparison with histidine and NA, metal-organic acid complexes are of lower thermodynamic stability, but less affected by low pH. Organic acids have long been implicated as chelators acting in metal tolerance and accumulation, but there is no direct evidence for their mechanistic importance to date. In the metal hyperaccumulators *N. caerulescens* and *A. halleri* Zn-citrate and Zn-malate are the most abundant Zn-ligand complexes [40,58]. In particular in the vacuoles of shoot tissues, where organic acid concentrations can reach very high levels, metals are thought to be stored in the form of organic acid-complexes [150,151].

### 3.13. Glutathione and phytochelatins (PCs)

Phytochelatins (PCs), small peptides synthesized enzymatically by Phytochelatin Synthase (PCS) from glutathione, are required for basal Cd tolerance in plants [152]. The *PCS1*-defictive *cad1* mutant of Arabidopsis is Cd-hypersensitive and, under some conditions, Zn-hypersensitive showing reduced accumulation of Zn in roots [54]. Similar to Cd exposure, Zn exposure can induce PC biosynthesis in *Schizosaccharomyces pombe*, and a Zn-sensitive strain became even more sensitive when the *PCS* gene was additionally disrupted. The ubiquity of *PCS* genes in the plant kingdom points to a role in essential metal homeostasis, although Arabidopsis *PCS1* appears to have acquired an additional role in indole glucosinolate catabolism [153]. Cytosolic concentrations of reduced glutathione are in the millimolar range [154], and – according to its binding properties – a fraction of it could act as an intracellular Zn ligand. Recent work has implicated the biosynthesis of glutathione, but not phytochelatins, in basal Zn tolerance [155], but this remains difficult to interpret due to the possible pleiotropic effects of glutathione deficiency.

### 3.14. Metallothioneins (MTs)

Metallothionein proteins are small, cysteine-rich proteins capable of binding transition metals. They are important in essential metal homeostasis and the detoxification of non-essential heavy metals in many organisms [156]. The seven genes encoding MTs in Arabidopsis fall into four different classes [152]. Plant MTs, however, exhibit only limited similarity to MTs of other organisms. Expression of all seven Arabidopsis *MT* cDNAs in yeast rescued the Cu-sensitive *cup1* mutant, which is lacking the yeast Cu metallothionein acting in cytoplasmic Cu buffering. Expression of *MT4a* and *MT4b* also rescued a Zn-sensitive yeast mutant. *MT1a* is implicated in root Cu homeostasis [156]. Recent data on *A. thaliana MT4a* and *MT4b* suggest that they are important in Zn storage in seeds. Expression levels of *MT4a* and *MT4b* correlate with Zn levels in the seed, and with the ability of the seed to germinate in low-Zn conditions [34].

MT functions have also been studied in metal hyperaccumulator plants. A modified structure of NcMT3 enables this protein to confer far higher Cu tolerance than AtMT3 to an *ace1* (=*cup2*) yeast mutant defective in the gene encoding the transcriptional activator of the yeast metallothionein gene *CUP1*. This was interpreted to suggest that a higher binding affinity for Cu might be required in metallothioneins of Zn hyperaccumulators [157]. *NcMT2a*, *NcMT2b* and *NcMT3* are expressed at much higher levels in *N. caerulescens* than their orthologues in *A. thaliana*. However, neither metal accumulation nor metal tolerance is enhanced in *A. thaliana* ectopically overexpressing *NcMT2a* or *NcMT3* [158]. The present status of knowledge suggests a primary role for these *MT* genes in maintaining Cu homeostasis in hyperaccumulators in the presence of elevated Zn and Cd levels.

## 4. Regulation of Zn homeostasis in the context of its interactions with other metals

In *S. cerevisiae*, Zn deficiency responses are controlled by ZAP1, a transcriptional activator binding to Zinc-Responsive Elements (ZREs) in the promoters of its target genes [159]. ZAP1 has a single DNA-binding domain and two activation domains that independently bind Zn, resulting in the inability to activate transcription. ZAP1 is autoregulatory, inducing its own expression under Zn deficiency [160]. Other key targets of ZAP1 are the promoters of the genes encoding yeast ZIP family transporters ZRT1 and ZRT2 that mediate cellular Zn uptake, and ZRT3 that remobilises Zn from vacuolar storage. Analogous responses to Zn deficiency are also of key importance in plant Zn homeostasis, and yet, no orthologue of ZAP1 has been identified in plants.

According to our present state of knowledge, transcriptional control contributes strongly to Zn homeostasis in plants. Transcripts of a number of *ZIP* genes, *NAS* genes and *HMA2* increase in response to Zn deficiency [90,63,161,162]. Comparisons of transcript levels of orthologous genes from Zn hyperaccumulators and non-accumulators under different Zn regimes have implicated a number of additional genes in the Zn deficiency response [86] (Fig. 1). Moreover, most known Zn deficiency response genes appear to be constitutively highly expressed in hyperaccumulators, although their physiological roles are not well understood [163]. This goes along with high expression of Zn homeostasis genes not associated with deficiency responses, encoding the vacuolar Zn transporter MTP1, and the Zn/Cd pumps HMA3 and HMA4. The deregulation of most, but not all, Zn deficiency response genes in Zn hyperaccumulators is a secondary consequence of symplastic Zn depletion through the strongly enhanced expression of *HMA4* [39] and, possibly, also *MTP1* [108] (Figs. 1 and 2).

Recent ground-breaking work in *A. thaliana* has employed regions of the *ZIP4* promoter in a yeast one-hybrid screen to identify two transcription factors, bZIP19 and bZIP23. These are able to bind to a specific Zinc-Deficiency Response Element motif (ZDRE: RTGTCGACAY) that is present in two copies in the promoter region of *ZIP4* [89]. A double *bzip19bzip23* mutant is hypersensitive to Zn deficiency, shows decreased root and shoot Zn accumulation and cannot accumulate Zn deficiency-responsive transcripts [89]. A microarray comparison between the Col-0 wild type and *bzip19bzip23* identified only the transcripts of 23 genes as being differentially regulated. Transcript levels of eleven of these genes are known to be upregulated in response to Zn deficiency, and nine genes, presumably direct targets for bZIP19/bZIP23, contain ZDREs in their promoter regions. Seven of these nine genes encode ZIP family transporters, suggesting that this regulatory system is largely specific for *ZIP* transporter and *NAS* genes [89]. Orthologues of bZIP19 and bZIP23 are found in rice, poplar and even in the moss *Physcomitrella patens*, and ZDREs were additionally reported in promoters of ZIP genes of rice and poplar, suggesting that this mechanism of regulating the response to Zn deficiency is widespread in vascular plants [89]. This study did not provide any insights into how a plant might sense Zn deficiency. The bZIP19 and bZIP23 proteins may additionally sense Zn, or alternatively, there are factors upstream that are involved in the sensing of Zn deficiency and the transmission of a signal to stabilize or activate the bZIP19 and bZIP23 proteins, as *bZIP19* and *bZIP23* transcript levels are unaffected by plant Zn status.

Nothing is known to date about whether Arabidopsis possesses mechanisms to economise on Zn similar to those that economise on

Cu [164]. The much higher biochemical requirement for Zn than for Cu might render such strategies unfeasible.

Vacuolar membrane proteins MTP1, MTP3, HMA3 and ZIF1 are all important in basal Zn tolerance, and, except for MTP1, the abundance of the transcripts encoding them increases in response to excess Zn as well as under Fe deficiency [87,81,25,53,36] (Fig. 3). An iTRAQ proteomic analysis confirmed that protein levels of the $Zn/H^+$-antiporter MTP3 in the vacuolar membrane increase under conditions of excess Zn [25,82]. Under Fe deficiency, the activation of the response to high Zn is important to compensate for the broad substrate specificity of the primary transporter contributing to root Fe uptake in Arabidopsis, IRT1, which is known to mediate the accumulation of a broad range of divalent transition metal cations including $Zn^{2+}$ [25,36,72]. The coordinated regulation of transcript levels of *MTP3*, *HMA3*, *ZIF1*, *IRT1* and *FRO2* may represent a response that is commonly triggered by both Fe deficiency and excess Zn, or alternatively reflect a physiological state commonly caused by both growth conditions. As indicated above, Fe deficiency causes secondary Zn accumulation that is caused by increased IRT1 protein levels. Conversely, it is known that exposure to excess Zn causes physiological Fe deficiency. Both *MTP3* and *HMA3* transcript levels were identified as reduced in the *fit* mutant defective in the bHLH transcription factor that activates root Fe deficiency responses [71]. Recent work suggested that the increase in *MTP3* transcript levels is a secondary consequence of *IRT1*-dependent root Zn accumulation under Fe deficiency, whereas the increase in *ZIF1* transcript levels under Fe deficiency is independent of Fe deficiency-induced Zn accumulation [25,36]. This provides a first glimpse of the complexity of the regulatory cross-talk between Fe deficiency and Zn excess (see Fig. 3). In the future, it will be important to dissect the common and metal-specific elements of the regulatory pathways governing these responses.

According to earlier studies, IRT1 protein appeared to be absent in Arabidopsis grown in the presence of excess Zn [76] (Ute Krämer, unpublished data), suggesting that the known regulation of IRT1 protein levels through ubiquitin-mediated proteasomal degradation [77,78] might predominate under this condition. However, recent studies provided evidence for the accumulation of increased levels of IRT1 protein in Arabidopsis grown under excess Zn conditions when compared to plants grown under control conditions [55,82] (Fig. 3), which would contribute dramatically to Zn toxicity. It will be interesting to see how this major discrepancy can be resolved. Additional sites of interference between movement pathways and binding sites of Zn and Fe in plants are likely to be identified in the future.

It is thus becoming increasingly clear that Zn homeostasis cannot be addressed without considering its interactions with other metals, in particular Fe (see Fig. 3). In addition, transcriptomic data suggest that, in common with Zn deficiency, Cu deficiency can also trigger increases in *ZIP2*, *ZIP4*, *ZIP5* and *NAS2* transcript levels. Moreover, the chemical similarity between Zn and Cd implies that the non-essential metal Cd may interact with or interfere with Zn homeostasis. This is supported by the fact that many Zn hyperaccumulators are also Cd hyperaccumulators, implying commonalities between the molecular and physiological mechanisms for Zn and Cd hyperaccumulation [29]. It has also been shown that *A. thaliana HMA2* and *HMA4* function in root-to-shoot translocation of both Zn [26] and Cd [102].

## 5. Signalling in Zn homeostasis

Factors involved in uptake and utilisation of a number of nutrients are now known to be regulated systemically, thus involving mobile signals [165]. Split-root experiments in Arabidopsis and barley have shown that the regulation of the expression of *NRT2* genes encoding transporters that contribute to nitrate uptake by the root is influenced by shoot nitrate status [166]. Conversely, the abundance of transcripts encoding ammonium transporter AMT1.1 of Arabidopsis is regulated in shoots in response to the ammonium level in roots [167]. The mechanisms responsible for the transmission of these signals are unknown. It has been proposed that under phosphate deficiency, a miRNA may constitute a mobile signal involved in shoot-to-root signalling of P status [168]. In response to P deficiency, the upregulation of miR399 levels in shoots and miR399 translocation *via* the phloem leads to a reduction in the levels of *PHO2* transcript in roots, as shown in grafting experiments [168,169]. The PHO2 protein is an ubiquitin-conjugating E2 enzyme that regulates root-to-shoot translocation of P by suppressing the loading of P into the xylem in roots. Responses to other nutrients may be controlled by similar signalling events. Other potential signals in nutrient homeostasis might involve hormones, in particular auxin. The role of auxin in controlling lateral root formation has been known for some time [170], and alterations in lateral root formation occur when plants are deficient in a variety of nutrients [171–173]. Localized lateral root elongation in Fe deficient plants upon Fe re-supply was recently shown to be auxin-dependent [174].

Evidence for long-distance systemic signalling of metal status has come from studies on Fe homeostasis. Split-root experiments, in which half the root system of a plant was placed in Fe-deficient hydroponic medium and the other half was placed in Fe-replete medium, showed that *IRT1* and *FRO2* transcript levels, both of which are central in the Fe deficiency response, are regulated by both local Fe levels in the apoplast and by a shoot-derived systemic Fe deficiency signal [72]. This supports earlier reciprocal grafting work in *Pisum sativum* using a mutant impaired in Fe homeostasis, *dgl*, which showed that the genotype of the shoot determines the phenotype of the root with respect to the activation of the Fe acquisition machinery [175]. Experiments in *Nicotiana tabacum* also support a role for a long-distance signal in the regulation of Fe uptake. Application of Fe(II)-EDTA to the leaves, as well as removing the leaves, of Fe-deficient *N. tabacum* plants resulted in the repression of *NtIRT1* and *NtFRO1* transcript levels in the roots [176]. This supports that a shoot-to-root Fe-deficiency signal critical for transcriptional iron deficiency responses of roots is generated in the leaves.

Systemic signalling in Zn homeostasis has hardly been addressed to date. When shoot scions of *A. thaliana* plants overexpressing *NgMTP1* were grafted onto wild-type roots, Zn-deficiency responses were observed in both roots and shoots of these grafted plants [108] (Fig. 4). Because the authors did not include root Zn concentrations, it is unclear whether the presented increase in *ZIP3* and *ZIP9* transcript levels occurred in response to local or systemic cues. These root Zn deficiency responses could be a secondary consequence of the activation of a systemically regulated factor driving xylem loading of Zn in the root, thus resulting in Zn depletion inside the root symplasm (in analogy with [39]). In this case, the upregulation of *ZIP3* and *ZIP9* transcript levels would be a response to a secondary, locally generated Zn deficiency signal. If, instead, the roots of these plants were Zn replete, this study would indicate that *ZIP3* and *ZIP9* are direct targets of a systemic Zn deficiency signal. It should be noted that both models presented here implicate a systemic signal communicating shoot Zn status to the root. A potentially interesting tool to further investigate the systemic regulation of Zn homeostasis in plants is the Arabidopsis *hma2hma4* double mutant, in which the root is known to be Zn-replete whereas the shoot is Zn-deficient [26]. Comparative transcript profiling in this mutant and the wild type will allow an advanced analysis of the systemic Zn deficiency response.

## 6. Outlook

Heterologous screening and expression in yeast mutants were the first and most prominently used approaches to identify and functionally characterize plant cDNAs acting in Zn homeostasis. Further approaches used extensively were the characterisation of overexpression lines and mutants obtained in forward and reverse genetics approaches, examining Zn localisation, site-directed mutagenesis to determine metal-binding

sites, microarray expression analysis after exposure to excess Zn or to Zn deficiency, and comparisons between the model plant *A. thaliana* and Zn/Cd hyperaccumulators *A. halleri* and *N. caerulescens* [31,26,177,18,178,30,179,28,82,29].

Future work might emphasize yeast-1-hybrid approaches employing the promoters of Zn status-responsive genes that are not regulated by bZIP19/bZIP23, such as *HMA2* or *ZIF1* [26,53]. This may allow the identification of additional transcription factors that have regulatory roles in Zn homeostasis. Yeast-2-hybrid screens and other techniques for the analysis of protein-protein interactions will be important in determining whether the activities of Zn membrane transporters or Zn-binding proteins are modulated by protein-protein interactions, and also in identifying binding partners for bZIP19 and bZIP23 that may influence the Zn deficiency response.

The biochemistry of factors involved in Zn homeostasis is not well understood, partly because obtaining crystal structures of large integral membrane proteins is very difficult. Work on AtHMA2 determined important residues in Zn and Cd binding. The binding of $Zn^{2+}$ to the N-terminus results in structures resembling those observed in Cu-transporting P-type ATPases when Cu(I) is bound to the N-terminus, and this was implicated in the activation of the transporter [180]. The extended His- and Cys-rich C-terminal cytoplasmic domain of HMA4 has a significant capacity to bind $Zn^{2+}$ and $Cd^{2+}$, and it confers Zn and Cd tolerance to yeast [181]. The physiological relevance of this in plants, however, is unclear. A stretch of histidine residues in the C-terminus of *N. caerulescens* HMA4 has been used as a natural His-tag, allowing purification of the transporter by taking advantage of its high abundance in this metal hyperaccumulator species. Biochemical characterization was then possible, allowing the determination of enzyme activation by Zn, Cd and Cu [182]. Further advances in the expression and purification of membrane proteins may allow a better understanding of the mechanisms of Zn transport, as well as the possible interactions between membrane transporters and compounds acting in cellular Zn trafficking.

The number of tools available for reliable imaging and localisation of Zn within plant tissues and cells is expanding. Synchrotron X-ray Fluorescence techniques have recently been employed to image metals including Zn in plant tissues, in particular in Arabidopsis seeds [183,177,184]. The use of these techniques has also been extended to other species, including Zn hyperaccumulators [185]. This work has contributed important knowledge of the localisation of metals in hyperaccumulating plants and of the physiological roles of specific metal homeostasis genes in Arabidopsis. To date, these techniques are not universally applicable and unable to resolve *in vivo* cellular dynamics of metal concentrations and availabilities. The use of chemical fluorophores to image Zn in plant cells through confocal microscopy has allowed the characterisation of alterations in metal homeostasis in mutant and transgenic plants [18,39]. Recently, the development of genetically-encoded ratioable FRET-based Zn sensors has shown promise in monitoring intracellular Zn homeostasis. These sensors can be targeted to particular organelles, allowing the monitoring of Zn within different cellular compartments [20]. Adaption of these developments

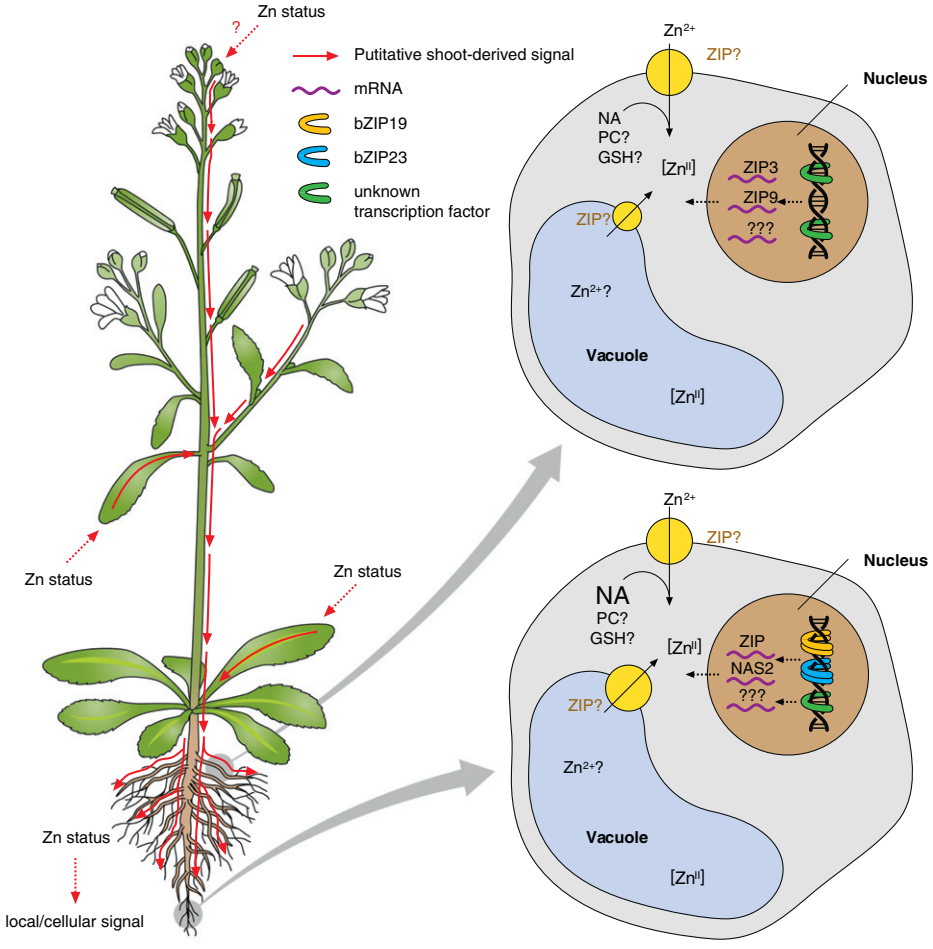


**Fig. 4.** Potential signalling of Zn deficiency. Shown is a hypothetical model of proposed systemic (upper left and upper right) and local (lower left and lower right) responses to Zn deficiency in root cells. Increases in *ZIP3* and *ZIP9* transcript levels were observed in roots when *NgMTP1* was overexpressed in shoots (hypothesized to generate shoot-specific physiological Zn deficiency; [108]). This supports the existence of a putative systemic Zn-deficiency signal originating from the shoot that triggers Zn deficiency response gene expression in the root through unknown regulators. Local Zn deficiency results in the activation of bZIP19 and bZIP23, which activate the transcription of a number of *ZIP* and *NAS* genes [89] (see also Fig. 1). Additional regulators may mediate further components of the Zn deficiency response. See main manuscript text for a detailed discussion.

for use in plants would allow the monitoring of Zn in living plant cells and tissues, and provide important information on the dynamics of Zn homeostasis in different cellular compartments.

In summary, great progress has been made in identifying and understanding the roles of individual proteins and chelators contributing to plant Zn homeostasis. However, further work is needed to develop a functional model of the plant Zn homeostasis network at both the cellular and whole-plant levels.

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